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ORIGINAL ARTICLE

Systematical approach in evaluation of LC method for determination of raloxifene hydrochloride and its impurities employing experimental design

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Abstract Method validation presents a detailed investigation of analytical method and provision of the evidence that the method, when correctly applied, produces results that fit to the purpose. In order to achieve the method validation scope efficiently, experimental design presents a very useful tool. The greatest benefits of such approach could be seen in robustness testing through the provision of very useful data about the control of the chromatograp^{6}hic system during the routine application. In this paper, robustness testing of the LC method proposed for the determination of raloxifene hydrochloride and its four impurities was done employing Plackett–Burman design. Applying this design, the effect of five real factors (acetonitrile content, sodium dodecyl sulfate content, column temperature, pH of the mobile phase and flow rate) on the corresponding resolution factors was investigated through twelve experiments. Furthermore, the insignificance intervals for significant factors were calculated and the parameters for system suitability tests were defined. Eventually, the other validation parameters were tested and the effectiveness of the proposed analytical method with a high degree of accuracy was confirmed.

1. Introduction

Method validation can be defined as the process of establishing the performance characteristics and limitations of a method, as well as the process of identifying the influences that may change these characteristics and to which extent [1]. In general, validation should check that the method performs adequately for the intended purpose through the whole range of analyte concentrations to which it is applied. In modern pharmaceutical analysis it is recommendable to

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support method validation by the application of appropriate experimental design, especially in the area of chromatographic analysis. There are many review papers dealing with the description of experimental design usage in chromatography and one of the latest is briefly discussing all kinds of designs [2]. In general, method development and validation supported by experimental design have many advantages over traditional approach one-factor-at-the-time. The main reason is the extraction of many useful data and drawing plenty important conclusions from relatively small number of well planned experiments. The significance of experimental design application could be seen in robustness testing which presents an integral part of method validation. Some recommendations and examples of robustness testing are given in the literature [3–6].

In this paper, presented approach is applied in robustness testing of reverse phase high performance liquid chromatographic (RP-HPLC) method for the analysis of raloxifene hydrochloride and its four impurities. Raloxifene hydrochloride is chemically [6-hydroxy-2-(4-hydroxyphenil)benzo[b]thien-3-yl]-[4[2-(1-piperidinyl) etoxy]phenyl]methanone hydrochloride and its four structurally related impurities are 2-(4-hydroxyphenyl)-1-benzotiophene-6-ol (impurity 1), piperidyl ethoxy benzoic acid (impurity 2), dimesyl benzotiophene (impurity 3) and raloxifene mesylate (impurity 4). Their structures are presented in the Fig. 1.

Literature survey showed many papers dealing with isocratic or gradient HPLC analysis of raloxifene in pharmaceuticals [7–14]. Recently, the analysis of raloxifene employing ultra-performance liquid chromatography (UPLC) with isocratic elution was published [15]. On the other hand, there is very limited number of papers where impurities of raloxifene are investigated. In one of the papers, only the presence of N-oxide was investigated [16]. Furthermore, in the paper by Chandorkar et al. only one impurity was tracked [17]. Finally, for the first time, raloxifene became official in Ph. Eur. 7 [18] where the method for the LC analysis of the related impurities is proposed. For the determination of impurity A and other unspecified impurities a gradient elution mode method was proposed to be conducted on the base-deactivated octylsilyl silica gel column with a mobile phase containing acetonitrile and potassium dihydrogen phosphate solution which pH was adjusted to 3.0 with phosphoric acid. At this point it is very important to note that the impurities analyzed in our paper are structurally different from those given in Ph. Eur. 7. The only paper dealing with the chromatographic analysis of those impurities is our previous publication mainly focused on the evaluation of the new chromatographic function (N_{CRF}) [19]. Eventually, the papers dealing with the analysis of raloxifene in biological samples could also be found in literature [20–23].

Finally, the aim of this paper was to present the improvement in method evaluation. Firstly, the development of back up method for the leading method was presented. Secondly, this method was thoroughly tested, especially its robustness, by the application of experimental design. The proposed method is intended for the determination of raloxifene hydrochloride and its listed impurities, so the validation studies are conducted to provide a realistic survey of the effects that might influence the method during its normal use.

2. Experimental

2.1. Chemicals

All used reagents were of the analytical grade. The mobile phase and the solvents were prepared using acetonitrile (Lab Scan, Ireland), ortho-phosphoric acid (Carlo Erba, Italy), sodium dodecyl sulfate – SDS (Sigma-Aldrich Chemie, GmbH, Germany) and HPLC grade water.

2.2. Chromatographic conditions

The experiments were performed on the chromatographic system Finnigan Surveyor Thermo Scientific which consisted of an HPLC pump, an autosampler plus and a UV/vis plus detector. ChromQuest was used for data collection. The analytical columns used in this study were XBridge C_{18}(3 mm × 100 mm, 3.5 µm particle size) and SunFire C_{18} (3 mm × 100 mm, 3.5 µm particle size). The mobile phase composition was acetonitrile:water phase, where the water phase consisted of SDS, while pH of the water phase was adjusted with ortho-phosphoric acid. The other chromatographic conditions were flow rate 1 mL/min, column temperature 35 °C and UV detection at 254 nm.

Mixture of acetonitrile:water phase in the ratio 44:56 (v/v) was used as solvent. Water phase was 6 mM SDS in water with pH adjusted to 4.5 with ortho-phosphoric acid.

2.3. Standard solutions

Stock solutions of raloxifene hydrochloride and the impurities were prepared by dissolving them in the solvent to obtain the

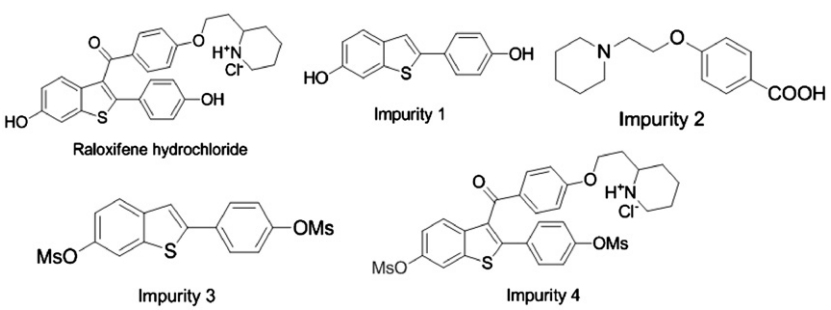

Fig. 1 Chemical structures of raloxifene hydrochloride and its impurities.

concentration of 100 μg/mL for raloxifene hydrochloride and 0.5 μg/mL for all impurities. The prepared stock solutions were stored at 4 °C.

2.4. Experimental data set for robustness testing

Twelve experiments defined by Plackett–Burman experimental plan were performed by varying the five real factors and six dummy factors around the nominal level. The included real factors and its intervals were acetonitrile content in the mobile phase (from 43% to 45%), sodium dodecyl sulfate content in water phase (from 5.5 mM to 6.5 mM), column temperature (from 40 °C to 50 °C), pH of the mobile phase (from 4.3 to 4.7) and flow rate (from 0.9 mL/min to 1.1 mL/min). Values given in the brackets present their low (−1) and high (+1) levels.

2.5. Solutions for validation

2.5.1. Solutions for linearity estimation

To evaluate the linearity of the developed method, seven solutions of raloxifene hydrochloride in the concentration range from 10 μg/mL to 120 μg/mL and seven solutions of impurities (Imp. 1–Imp. 4) in the concentration range from 0.05 μg/mL to 0.6 μg/mL were in the solvent using the appropriate standard solutions.

2.5.2. Solutions for accuracy estimation

The accuracy of the method was proved by preparing three series of solutions containing appropriate placebo (contains all substances from tablets except active substance and impurities), raloxifene hydrochloride, and impurities 1–4 in the solvent. These mixtures were prepared in three levels: (a) low level 80%: containing 80 μg/mL of raloxifene hydrochloride and 0.4 μg/mL of the impurities; (b) medium level 100%: containing 100 μg/mL of raloxifene hydrochloride and 0.5 μg/mL of the impurities and (c) high level 120%: containing 120 μg/mL of raloxifene hydrochloride and 0.6 μg/mL of the impurities.

2.5.3. Solutions for precision estimation

To prove the precision of the method, six identical solutions of powdered tablets in the water phase containing 100 μg/mL of raloxifene hydrochloride were prepared. The appropriate volume of impurities stock solutions were added to all solutions in order to obtain the final concentration of 0.5 μg/mL.

2.5.4. Sample solutions

Pulverized tablet mass containing 25 mg of raloxifene hydrochloride was extracted with the solvent in 25 mL volumetric flask placed in ultrasonic bath for 15 min. After filling the flask to the mark, the solution was filtered. This stock solution was used to prepare six solutions containing 100 μg/mL of raloxifene hydrochloride.

3. Results and discussion

When HPLC method is used for the simultaneous analysis of active substance and its impurities many different aims have to be achieved. One of the most important aims is reaching an unambiguous separation among analyzed substances that are usually structurally very similar. The second one is the acquirement of their precise and accurate quantification. For the most efficient meeting of the defined aims careful preliminary study should be conducted. As it could be seen from Introduction part, there are no papers dealing with the simultaneous determination of raloxifene hydrochloride and its listed impurities. In our previous paper [19], this mixture was used for the definition and evaluation of new chromatographic function. During this study, XBridge C_{18} (3 mm × 100 mm, 3.5 μm particle size column) was used and under the chromatographic conditions stated in that publication appropriate separation was achieved. However, new trends in pharmaceutical analysis suggest setting of a back-up method. The experiments were conducted on SunFire C_{18} (3 mm × 100 mm, 3.5 μm particle size column) and the acceptable separation was obtained. Basically, for the separation, on both columns, the mobile phases with acetonitrile or methanol were tested and the changes in organic modifier content were followed by the changes of water phase content. As a water phase, various buffer systems as well as ion pairing reagents were investigated. pH value of the water phase was tested in the range from 2.5 to 5.0. From a certain number of experiments some conclusions were extracted: (a) on both columns the optimal chromatographic separation could be attained; (b) acetonitrile was a better choice as the organic solvent as it affected positively on the peak symmetry, shortened the run time, etc.; and (c) ion pairing reagent is desirable in water phase as it enabled the adequate separation of raloxifene hydrochloride and the analyzed impurities. Finally, as the most suitable, the following chromatographic conditions were chosen:

SunFire C_{18} (3 mm × 100 mm, 3.5 μm particle size), acetonitrile–water phase (6 mM SDS, pH 4.5 adjusted with ortho-phosphoric acid) 44:56 (v/v), flow rate of 1 mL/min, temperature of 45 °C, and detection wavelength of 254 nm.

XBridge C_{18} (3 mm × 100 mm, 3.5 μm particle size), acetonitrile–water phase (4 mM SDS, pH 2.5 adjusted with ortho-phosphoric acid) 47:53 (v/v), flow rate of 1 mL/min, temperature of 35 °C, and detection wavelength of 254 nm.

Amongst these two optimal methods the first one was chosen for the further testing and the second one was denoted as a back-up method. This means, if further tests would show that the selected method would not pass the requirements, then all tests should be conducted with the back-up method.

In the next step, the method's robustness evaluation was done using Plackett–Burman design. Theory of Plackett–Burman design is given by Vander Heyden et al. [4] and we have already used this design for robustness testing in the method validation [5,24]. So, in this paper only data important for the analyzed case are given. According to the observations during the preliminary study, the previous experience and the knowledge in robustness testing of LC methods, acetonitrile content, sodium dodecyl sulfate content, column temperature, pH of the mobile phase and flow rate were selected as investigated factors. The Plackett–Burman design was completed by the addition of six dummy factors. Dummy variables are imaginary factors whose changes do not affect the system. They are added in order to provide statistical evaluation of the results and their changes from −1 to +1 level do not have physical meaning. As outputs, resolution factors between

adjacent peaks were followed. Plan of experiments defined by Plackett–Burman design and the obtained results for resolution factors are given in Table 1. Factors effects and results for $E_{critical}$ and ME (margin of error) are calculated and presented in Table 2.

Statistical evaluation followed the graphical presentation using a half normal probability plot (Fig. 2A) and Pareto chart (Fig. 2B).

Namely, when creating the half-normal plots, the n effects are ranked in a sequence according to the increasing absolute size of the effect. Unimportant factors are those that have near-zero effects and important factors are those with effects considerably removed from zero. Thus, unimportant effects that tend to have a normal distribution centered near zero are normally distributed around the straight line, while the significant effects deviate from it. Despite the strong recommendation for using half-normal plots in selecting statistically significant effects, Pareto chart can sometimes be very effective [4]. In this case, results for Pareto charts were evaluated on the basis of t limits. All the results related to factor's influence are summarized in Table 3.

It is obvious that different approaches designated quite different factors as important. Therefore, the recommendation is to do two or more tests (statistical and graphical) and compare the obtained results. In this paper, we applied four methods and the results matched for several factors but the rest of the factors, especially the less important ones appeared as significant only in some tests. For the calculation of non-significant interval for significant variables, results from both statistical tests were included. For the definition of this region equation proposed in Ref. [4] was applied

$$\left[X_{(0)}-\frac{|X_{(1)}-X_{(-1)}|E_{\text{critical}}}{2|E_X|},\ X_{(0)}+\frac{|X_{(1)}-X_{(-1)}|E_{\text{critical}}}{2|E_X|}\right] \quad (1)$$

where $X_{(0)}$, $X_{(1)}$ and $X_{(-1)}$ are the real values of factor X at the levels (0), (1) and (−1), respectively. Results for non-significant intervals of the significant factors are given in Table 4.

Next step in the method evaluation is a development of the control strategy which usually means a definition of the requirements for the system suitability tests (SST) which would be carried out each time when the method is used.

Table 1 Plan of Plackett–Burman design and experimentally obtained results.

Run	A	d_1	C	d_2	E	d_3	G	d_4	d_5	K	d_6	Rs_1	Rs_2	Rs_3	Rs_4
1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	2.16	7.05	3.17	13.08
2	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	+1	2.52	10.21	0	15.42
3	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	−1	2.25	9.22	2.69	11.43
4	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	−1	4.07	8.74	0	16.80
5	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	−1	0.83	10.93	0	18.92
6	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	+1	2.58	10.96	0	13.04
7	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	+1	3.89	7.06	2.95	13.03
8	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	+1	1.88	8.63	3.59	11.97
9	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	−1	2.15	9.13	2.62	13.34
10	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	+1	1.91	11.59	0	17.49
11	+1	−1	+1	+1	+1	−1	−1	−1	+1	−1	+1	1.28	7.99	2.35	11.18
12	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	1.67	11.09	2.53	16.36

A, acetonitrile content (%); d_1, dummy 1; C, sodium dodecyl sulfate (SDS) content in water phase (mM); d_2, dummy 2; E, column temperature (°C); d_3, dummy 3; G, pH of the mobile phase; d_4, dummy 4; d_5, dummy 5; K, flow rate (mL/min); d_6, dummy 6; Rs_1, Rs_2, Rs_3 and Rs_4, resolutions between adjacent peaks.

Table 2 Factors effects and results for $E_{critical}$ and ME.

Factors	R_1	R_2	R_3	R_4
ACN (%)	0.007	2.41	2.47	4.00
Dummy 1	0.37	0.32	0.19	0.69
SDS (mM)	0.89	0.93	0.76	0.58
Dummy 2	0.22	0.25	0.58	1.00
Temperature (°C)	0.38	1.44	0.49	0.80
Dummy 3	0.46	0.23	0.16	0.72
pH of the mobile phase	1.29	0.33	0.57	0.99
Dummy 4	0.41	0.04	0.24	1.20
Dummy 5	0.27	0.03	0.46	0.26
Flow rate (mL/min)	0.03	0.14	0.40	0.95
Dummy 6	0.15	0.05	0.35	1.30
$E_{critical}$ ($\alpha=0.05$)	0.6446	0.3744	0.7049	1.8075
ME (0.975; m)	1.1986	0.8091	1.0068	3.2546

ME, margin of error.

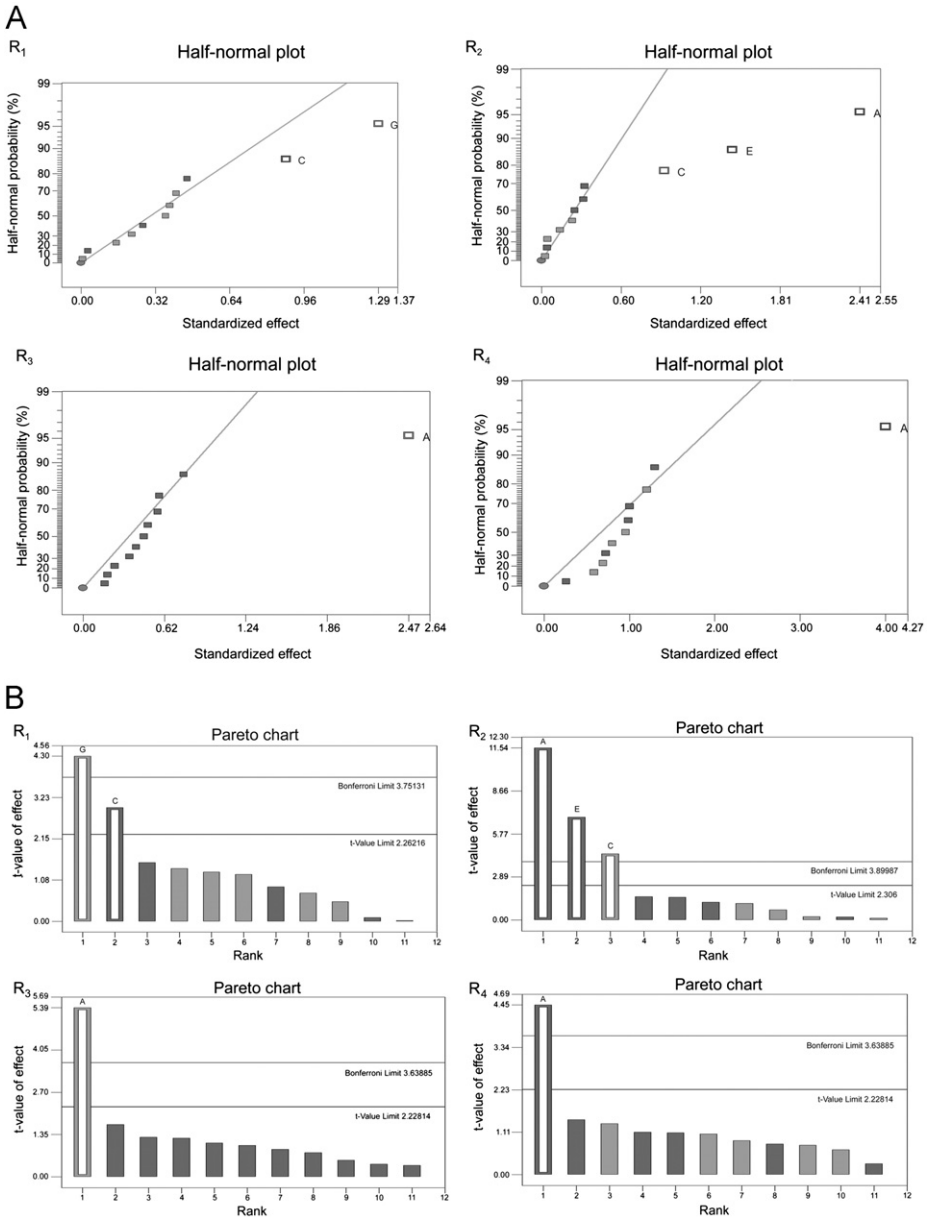

Fig. 2 Half-normal probability plots (A) and Pareto charts (B) for R_1, R_2, R_3 and R_4.

Table 3 Evaluation of factors effects.

Response	(a)	(b)	(c)	(d)
R_1	C+G	G	C+G	C+G
R_2	A+C+E	A+C+E	A+C+E	A+C+E
R_3	A+C	A	A	A
R_4	A	A	A	A

(a)Significant effects at $\alpha=0.05$ from comparation with critical effects from negligible effects
(b)Significant effects at $\alpha=0.05$ from algorithm of Dong
(c)Significant effects from the half–normal probability plot
(d)Significant effects from Pareto chart

One of the main advantages of the introduction of experimental design in robustness testing is the possibility to define SST using the worst case situations as it is recommended by Vander Heyden et al. [4]. These worst-case conditions could be predicted from the calculated effects. The worst-case situation is then the factors combination giving the worst

Table 4 Nonsignificant intervals for significant variables (dummy variable and Dong's methods).

Response	Significant factor	Nonsignificant interval obtained from $E_{critical}$ from dummies at $\alpha=0.05$	Nonsignificant interval obtained from $E_{critical}$ from Dong's algorithm at $\alpha=0.05$
R_1	C	5.64–6.36	-
	G	4.40–4.60	4.31–4.69
R_2	A	43.84–44.16	43.66–44.34
	C	5.80–6.20	5.56–6.43
	E	43.70–46.30	42.19–47.81
R_3	A	43.71–44.29	43.59–44.41
	C	5.54–6.46	-
R_4	A	43.55–44.45	43.19–44.81

A, acetonitrile content (%); C, sodium dodecyl sulfate (SDS) content in water phase (mM); E, column temperature (°C); G, pH of the mobile phase

Table 5 Important data for method validation.

Parameter	Raloxifene hydrochloride	Imp. 1	Imp. 2	Imp. 3	Imp. 4
Linearity range (μg/mL)	10–120			0.05–0.60	
Slope (a)	43486.57	76.57	75.02	68.70	71.86
Intercept (b)	−13.33	0.81	1.05	−0.49	0.83
Correlation coefficient (r)	0.9999	0.9997	0.9972	0.9992	0.9959
Accuracy given as recovery (%)					
Low level*	94.94±0.47	87.49±7.73	92.19±6.54	102.94±0.06	102.94±0.06
Medium level*	95.29±0.38	80.08±2.53	88.74±2.99	92.09±1.17	82.68±4.67
High level*	97.14±0.22	81.05±1.16	86.78±2.92	93.14±1.73	91.15±3.35
Precision RSD (%)	1.5	2.7	2.8	2.9	5.2

RSD, Relative Standard Deviation.
*Low level corresponds to 80%; medium level corresponds to 100%, and high level corresponds to 120%.

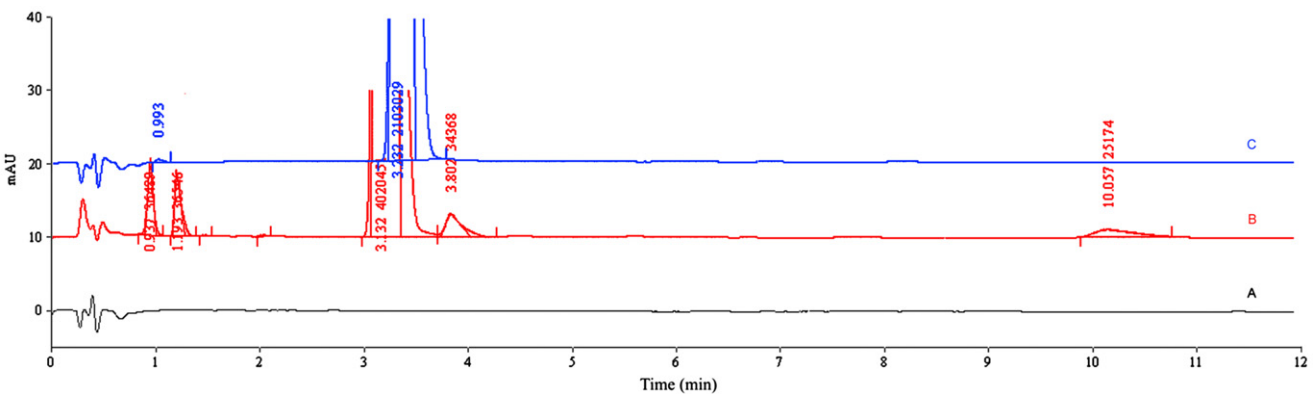

Fig. 3 Chromatograms of placebo (A), laboratory mixture (B) (0.937 min, Imp. 2; 1.193 min, Imp. 1; 3.132 min, raloxifene hydrochloride; 3.802 min, Imp. 3; and 10.057 min, Imp. 4) and analyzed sample (C) obtained under the optimal chromatographic conditions: SunFire C_{18} (3 mm × 100 mm, 3.5 μm particle size), acetonitrile–water phase (6 mM SDS, pH 4.5 adjusted with ortho-phosphoric acid) 44:56 (v/v), flow rate of 1 mL/min, temperature of 45 °C, and wavelength of 254 nm.

result for response. To define the worst-case conditions only the statistically significant factors (at $\alpha=0.05$) and the ones that come close to it (significant at $\alpha=0.1$) are considered [4]. Using Eq. (2) the worst result can be derived.

$$Y = b_0 + \frac{E_{F_1}}{2} * F_1 + \frac{E_{F_2}}{2} * F_2 + \ldots + \frac{E_{F_k}}{2} * F_k \quad (2)$$

where Y represents the response, b_0 is the average design result, E_{Fi} is the effect of the factor considered for the worst-case experiment and F_i is the level of this factor (–1 or +1). Non-important factors are kept at nominal value ($F_k=0$). Calculated values for SST are 1.625, 10.84, 4.075 and 16.34 for R_1, R_2, R_3 and R_4, respectively. It is obvious that only value for R_1 could be considered as critical and its values should be tested always.

After all in this investigations, the other parameters included in the method validation are tested and results are given in Table 5. This includes linearity, accuracy and precision testing, as well as the determination of limit of detection (LOD) and limit of quantification (LOQ) for the impurities.

Linear relationships of the peak areas vs. concentration for the concentration ranges mentioned above were obtained for raloxifene hydrochloride and all impurities. As the correlation coefficient (r) for the calibration curves of raloxifene hydrochloride and its impurities were greater than 0.9959 it can be concluded that the calibration curves were within the linearity acceptance criteria.

To evaluate the method accuracy, the recovery values for laboratory mixtures were calculated. Namely, certain amount of active substance and impurities were added to placebo and all procedure for tablets was followed in order to confirm that there are no interactions. Obtained values for all conducted tests are within the required values [25]. RSD values for precision were lower than 5% for impurities and 1.5% for raloxifene hydrochloride.

For the quantitative analysis of impurities it was important to define the values of LOD and LOQ. The signal-to-noise ratio of 3.3:1 and 10:1 were taken as LOD and LOQ, respectively, and further confirmed by taking dilutions from the secondary stock solution till the peak area obtained was 3.3 times (for LOD) and 10 times (for LOQ) bigger than the standard deviation of blank solution after six injections. The obtained values for all impurities were 0.025 mg/mL and 0.008 mg/mL for LOQ and LOD, respectively.

Chromatograms of placebo (A), laboratory mixture (B) and the analyzed sample (C) are presented in Fig. 3. Finally, tablets containing raloxifene hydrochloride were analyzed and content of raloxifene hydrochloride was 57.6 mg/tbl (96%) and Imp. 1 was 0.08%. Other impurities' contents (Imp. 2, Imp. 3 and Imp. 4), under validated chromatographic conditions, were below LOQ. So, it could be concluded that contents of all impurities are below maximal allowed level of 0.5%.

4. Conclusion

In this paper, validation of a new LC method proposed for the quantification of raloxifene hydrochloride and its four impurities was presented. The suitability of the method was confirmed by testing the appropriate performance parameters. In that way the realistic representation of the quality and reliability of the proposed method is acquired. Firstly, the robustness of the newly developed method was tested by the application of Plackett–Burman design. Statistical and graphical methods are used to designate the factors that influence method's robustness significantly. In order to enable the proper control of the proposed method during the routine application, non-significant intervals for significant factors and parameters for system suitability testing were defined. Also, all the other validation parameters were statistically evaluated and method's adequacy for the analysis of pharmaceuticals containing raloxifene was confirmed.

Acknowledgments

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